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A facile reactor process for producing 7,10-dihydroxy-8(*E*)-octadecenoic acid from oleic acid conversion by *Pseudomonas aeruginosa*[**]

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Abstract

Pseudomonas aeruginosa strain PR3 (NRRL B-18602) converts oleic acid to a novel compound, 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD). The bioconversion was scaled up in a 7-l bench-top, stirred-batch reactor to produce DOD for testing of potential industrial uses. Aeration was supplied continuously from the top through two ports on the headplate and periodically through a bottom sparger, in conjunction with the use of marine impellers for agitation. This unique aeration arrangement maintained the dissolved O_2 concentration in the 40–60% range during the period of maximal bioconversion and it also avoided excessive medium foaming during the reaction. Furthermore, the level of dissolved O_2 in the first 24 h of reaction played an important role in the initial rate of DOD production. DOD production reached a plateau after 72 h with a yield up to 100 g (or 50% recovery) from a total of 9 l medium from two reactors run simultaneously. The final culture broth was processed using newly adapted procedures in the pilot plant that included crystallization of DOD from ethyl acetate solution at −15 °C. The newly developed bioprocess will serve as a platform for the scale-up production of other value-added products derived from vegetable oils and their component fatty acids.

Introduction

A number of microbial systems have been examined for conversions of fatty acids in vegetable oils, especially soybean oil, to new products with enhanced functionality (Kuo *et al*. 2002). At the National Center for Agricultural Utilization Research, one of the most studied conversion reactions involves *Pseudomonas aeruginosa* strain PR3 (NRRL B-18602), which converts oleic acid (OA) to a novel compound, 7,10-dihydroxy-8(*E*)-octadecenoic acid (DOD) (Hou & Bagby 1991, Kuo *et al*. 1998). The maximum production of DOD with a yield of 63% was achieved in small volume (30 ml) cultures at 30 °C and 200 rpm in 48 h of the conversion reaction (Hou & Bagby 1991). Recently, optimization studies improved the production yield to 89% and demonstrated that Mn^{2+} was important for the bioconversion of oleic acid to DOD (Kuo *et al*. 1998, 2001). Furthermore, strain PR3 does not accumulate a hydroperoxide intermediate, 10-hydroperoxy-8(*E*)-octadecenoic acid, in the fermentation culture as does strain 42A2 (Guerrero *et al*. 1997). Our results are consistent with the DOD formation that involves 10(*S*)-hydroxy-8(*E*)-octadecenoic acid as an intermediate (Kim *et al.* 2000).

DOD is known to reduce surface tension (Para *et al.* 1990, Knothe *et al.* 1995) and it has been reported to inhibit growth of *Candida albicans* (Hou 2001). To further evaluate DOD for new industrial uses, an effective, large-scale process for producing DOD is needed. Herein, we report the results of developing a facile, scaled-up bioprocess for producing

[**]Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

DOD from technical grade oleic acid by strain PR3 in a bench-top, stirred-batch reactor.

Materials and methods

Materials

Oleic acid (technical grade at 90% purity) was from Aldrich. As it contained small amounts of palmitic acid, margaric acid (17:0) was used as an internal standard. The reference standards (>99.9%) were from Nu-Chek Prep, whereas DOD was purified as described previously (Kuo *et al.* 1998). Antifoam SO-25, the trade name for a polymeric polyol: silicone defoamer, was obtained from Sigma. All other chemicals were reagent-grade and used without further purification.

Microorganism and growth

Pseudomonas aeruginosa strain PR3 (NRRL B-18602; see Hou & Bagby 1991) was grown in 100 ml TGY medium, pH 7.2, which contained (g l^{-1}) 5 tryptone, 1 glucose, 5 yeast extract, and 1 K_2HPO_4 in a 250 ml Erlenmeyer flask at 28 °C and 200 rpm for 24 h. The culture was used to inoculate (1% v/v) 4.5 l SM6 medium (Kuo *et al.* 2001) containing 0.1 g Antifoam SO-25 l^{-1} in a 7-l bench-top fermenter (Applikon Inc., Foster City, CA). The fermenter was equipped with two three-bladed marine vortex impellers (60 mm diam.) for agitation and sensors for pH, temperature, and dissolved O_2 (DO) concentration as the readings were monitored by PC software and a model ADI 1030 biocontroller. The inoculated culture was grown for 7–10 h at 28 °C and 650 rpm.

Production of DOD in stirred batch reactor

The production of DOD was initiated by adding 100 ml technical grade oleic acid to the culture. The pH was subsequently adjusted to neutral by adding 6 M KOH dropwise over 30 min. Aliquots (10 ml) in three replications were taken daily for GC analyses to determine the lipid profile of the fermentation culture and the yield of DOD production.

During the bioconversion reaction, DO concentration was regulated by a newly designed aeration mechanism by which a filtered airflow was constantly supplied from the top through two ports on the headplate at 220 ml min^{-1} and, when needed, through a bottom sparger at varied rates as regulated by Aalborg flowmeters (Orangeburg, NY), model 082-03S and 062-01S, respectively. Such an aeration control system was needed not only to provide adequate DO concentrations to the culture but also to avoid excessive foaming from occurring during the production of DOD.

Lipid extraction and analysis

The samples that included both cells and the broth were acidified to pH 2 and extracted with 30 ml methanol/ethyl acetate (1:9, v/v). Solvents were evaporated from the combined extracts with a rotary evaporator. Methyl esters were prepared with diazomethane and analyzed by GC using the conditions described previously (Kuo *et al.* 2001). The yield of DOD production was calculated from GC percentages relative to 17:0, which was added as an internal standard prior to lipid extraction.

Processing of culture broth in pilot plant

The final culture broth including cells was acidified to pH 2 by adding 6 M HCl and extracted with ethyl acetate (1:2, v/v) using a newly designed apparatus as described in Figure 1. A second extraction on the culture broth was carried out using 1:1 (v/v) ratio. The extracts were combined (about 8 l) and reduced to less than 500 ml at 40 °C using a rotary evaporator. The product was crystallized out from ethyl acetate at −15 °C. DOD crystals were collected and rinsed with cold hexane to remove any remaining oleic acid. The crystallization step was repeated two to three times. The final product was subjected to melting point determination and GC analysis.

Results and discussion

Production of DOD from oleic acid by *P. aeruginosa* strain PR3 was initially investigated in 30 ml medium in 125 ml Erlenmeyer shake flasks (Hou & Bagby 1991, Kuo *et al*. 1998). In this study, the production was scaled up to obtain sufficient DOD for testing of new industrial uses. Preliminary experiments conducted in shaken flasks showed that addition of 0.1 g Antifoam SO-25 l^{-1} to the medium enhanced the bioconversion reaction. However, owing to the surfactant nature of DOD produced in the reactor, continued addition of the defoamer to the fermentation culture was unable to control excessive foaming generated during

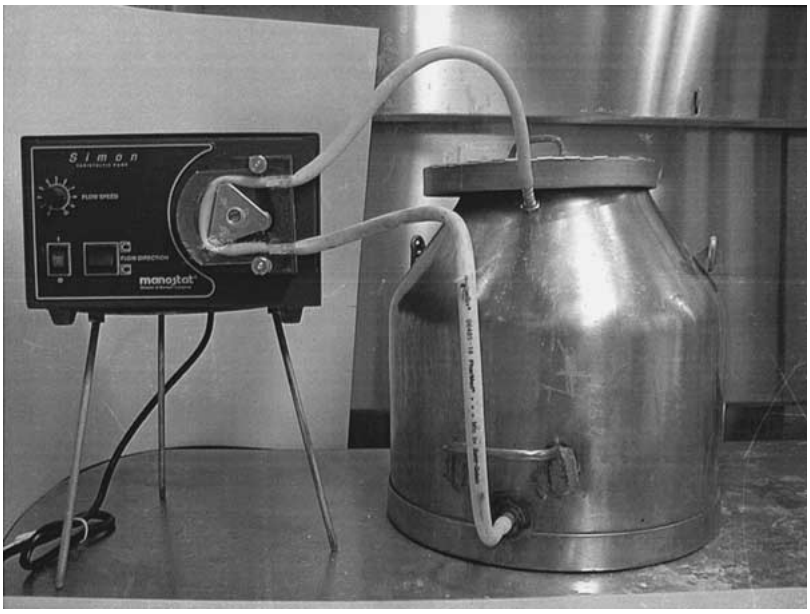

Fig. 1. A solvent extraction device that consisted of a varistalic pump and a stainless steel drum container. The 20-l stainless steel drum with a unique shape to reduce solvent splashes had two outlets connected by a pump tubing (0.5 cm ID). The lower outlet connector was parallel, whereas the upper inlet connector had a 45° angle to the bottom of the container. Solvent/culture broth including cells (2:1, v/v) was pumped from the lower outlet connector and into the upper inlet connector of the container. The pump was set at 1,25 l min^{-1} for 10 min to generate adequate extractions of the culture broth. Each batch of solvent after extraction was reused in the next batch of extraction to reduce the total volume of solvent used.

the reaction. The typical aeration through a bottom sparger was replaced by a new arrangement that combined a constant airflow from two ports on the headplate and, when needed, through a bottom sparger, in conjunction with the use of marine impellers for agitation. This unique aeration arrangement was able to maintain DO concentrations in the 40–60% range and avoid foaming from becoming uncontrollable during the conversion reaction. Fabritius *et al.* (1996) also applied an airstream blown from the top of a fermenter and a large, custom-made stirrer to control foaming during the production of 3-hydroxy-Δ^9-*cis*-1,18-octadecenedioic acid from oleic acid transformation by *Candida tropicalis* mutant strain M 25.

The formation of DOD by strain PR3 likely requires O_2 for carrying out the hydroxylation of oleic acid (Kuo *et al.* 2002). By varying parameters, such as the rate and the frequency of airflow passing through a sparger and the impeller's agitation speed, different levels of DO could be achieved to affect DOD production. As the impeller's agitation speed was held at 650 rpm and the airflow from the top of the headplate was held constantly at 220 ml min^{-1} and that through a bottom sparger held at a constant rate and frequency as shown in Figure 2A, the level of DO and the amount of DOD produced in the reactor broth were rising at a similar rate for the first 48 h of bioconversion. Thereafter, the DO concentration began to level off and decreased gradually to around 30% after 65 h, whereas DOD production continued to rise to 36 g after 4 d bioconversion. Meanwhile, the amount of oleic acid decreased most rapidly to about 36 g in the first 24 h and slowly in the next 3 d of reaction leaving a small amount detected in the reactor broth (Figure 2A). The results indicate that DO concentrations in the reactor broth during the first 24–48 h bioconversion is directly related to the yield of DOD production.

A simple modification of aeration conditions subsequently was made as shown in Figure 2B to increase the DO concentration to around 60% in the first 24 h of bioconversion. DOD production increased faster and reached a plateau earlier than the previous case of reaction (Figure 2A). However, the maximum production yield after 4 d bioconversion was similar in both cases. Examining the amounts of DOD and oleic acid in the reactor broth revealed that more than 80% of the added substrate disappeared in 24 h and the sum of DOD and oleic acid was about 40% of the total amount of substrate added to the reactor broth (Figure 2B). This indicates that a substantial amount of

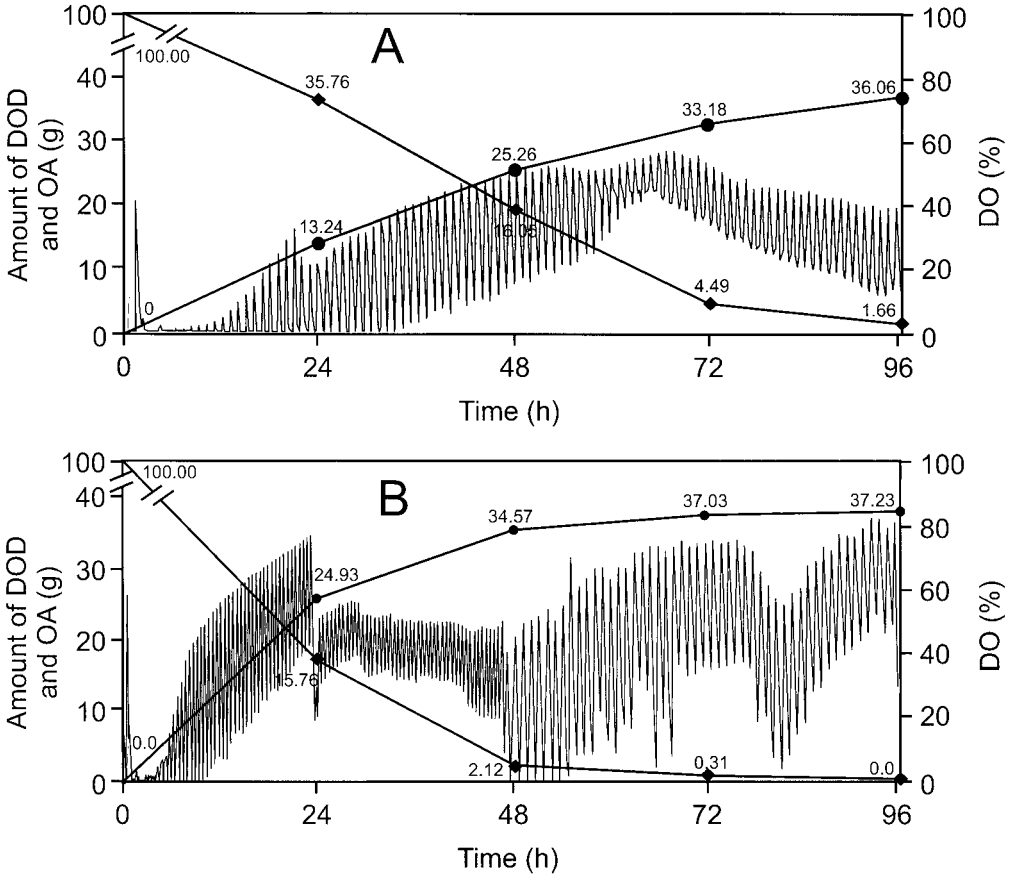

Fig. 2. A topographical illustration of changes in DO concentrations as related to DOD formation (●) and oleic acid disappearance (♦) during bioconversion in a 7-l reactor. (A) The conversion reaction was carried out in 4.5 l with 8-h-old *P. aeruginosa* strain PR3 at 28 °C under 650 rpm impeller speed, 220 ml min^{-1} airflow from top of the headplate and a bottom sparger airflow at 15 ml min^{-1} for 15 min h^{-1} throughout the entire process. The bioconversion was initiated by adding 100 ml technical grade oleic acid to the reactor culture that was subsequently adjusted to neutral pH. (B) Same conditions as (A) were used except that the sparger aeration rate was changed from 15 ml min^{-1} to 70 ml min^{-1} as the DO concentration decreased to about 20% in the final hour of cell growth, from 75 ml min^{-1} to 35 ml min^{-1} 24 h after adding oleic acid substrate, and finally from 35 ml min^{-1} to 15 ml min^{-1} concomitant with the sparger airflow frequency changed from 15 min in 30 min to 15 min h^{-1} after 48 h bioconversion.
DOD is 7,10-dihydroxy-8(*E*)-octadecenoic acid, OA = oleic acid, and DO = dissolved O_2. Each data point is the average of triplicate analyses.

oleic acid was utilized by strain PR3 probably as an energy source rather than being directly converted to DOD. Future research on minimizing the oleic acid consumption by strain PR3 is critical to achieve a higher yield.

Further decreases of the sparger airflow rate and frequency as described in Figure 2B were made after 24 h bioconversion. The changes maintained DO concentrations mainly in the 40–60% range as DOD production continued to increase to 34.6 g and reached a plateau after 72 h. The results suggest that DO concentrations in the first 24 h of bioconversion are most critical for the initial oleic acid conversion rate and the duration affecting the bioconversion to approach its maximum yield. Recently, Bastida *et al.* (1999) also reported a maximum amount of about 10 g 10-hydroxy-8(*E*)-octadecenoic acid (MHOD) was produced from oleic acid over 24 h by *Pseudomonas* sp. 42A2. The production of MHOD was positively related to the O_2 solution transfer coefficient, which is a function of the DO concentration present in the batch culture.

In a general laboratory practice, two identical cultures were prepared using 7-l bench-top fermenters. DOD production up to 100 g (a 50% yield) could be obtained from a total of 9 l culture broth in two reactors run simultaneously after oleic acid conversion for 4 d. The final culture broth was best ex-

tracted with ethyl acetate alone in a 1:2 (v/v) ratio by the practical Manostat-Varistalic-Pump projector device (Figure 1) to obtain a good separation of cell/aqueous medium from the extracted lipid fraction. The presence of methanol in a typical solvent mixture (1:9 v/v methanol/ethyl acetate) routinely used in our laboratory (Kuo *et al.* 2001) had extracted contaminants that showed inhibition to the subsequent crystallization of DOD. DOD crystals were readily formed from less than 500 ml ethyl acetate at −15 °C. The prepared DOD after 2–3 crystallization cycles had a melting point of 63–64 °C. However, the final product still contained a minor component (less than 1%), which is probably the intermediate [10(*S*)-hydroxy-8(*E*)-octadecenoic acid] synthesized during the formation of DOD (Kim *et al.* 2000).

In summary, a practical reactor process was applied to achieve a scale-up production of DOD from oleic acid conversion by *P. aeruginosa* strain PR3. This process involved the use of a new aeration mechanism to overcome the excessive foaming problems generated from the grown cell culture, the hydrophobic substrate, and the surface-active product in a stirred batch reactor. Furthermore, the bioproducts were processed using newly adapted procedures in the pilot plant and DOD was simply isolated by crystallization. Consequently, a facile reactor process is developed to provide DOD in hundred-gram quantity to researchers for testing new industrial uses. The newly developed technology will serve as a platform for further improvement on the scale-up production of other value-added bioproducts derived from vegetable oils and their component fatty acids.

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